Am. J. Potato Res. (2015) 92:49–61
DOI 10.1007/s12230-014-9410-z

Characteristics of Polish Isolates of *Fusarium sambucinum*: Molecular Identification, Pathogenicity, Diversity and Reaction to Control Agents

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Published online: 25 July 2014

Abstract Pathogenicity of 28 Polish isolates of *F. sambucinum* to potato tubers, their sensitivity to control agents, diversity among isolates and molecular methods of species identification were examined. All isolates were pathogenic to potato tubers and differences in pathogenicity were found. Isolates on the PDA were classified into three different color groups of mycelium (B - bright-beige, P - salmon pink, R - rose) that varied in pathogenicity and mycelium growth rate on PDA. P colonies showed the greatest tuber damage, but they grew the slowest on the PDA. Isolates showed varied reaction to different concentrations of 4 control agents (M - mancozeb, C- captan, CO - copper oxychloride and GE - grapefruit extract). The highest mycelium growth inhibition (MGI) was caused by M and the lowest by CO. Strong MGI by GE was observed especially for P isolates. Individual isolates showed different susceptibility to the control agents. Identification of isolates was determined in PCR assay with species specific FSF1/FSR1 primers, by sequencing of DNA fragments derived from ITS regions and the translation elongation factor-1 alpha gene (TEF). Sequence of the ITS regions were identical for all isolates. Analysis of the TEF DNA fragments showed one SNP (transition C↔T) in the sequences of isolates from the three different color groups.

Resumen Se examinaron la patogenicidad de 28 aislamientos polacos de *F. sambucinum* de tubérculos de papa, su sensibilidad a agentes de control, su diversidad entre aislamientos y métodos moleculares de identificación de especies. Todos los aislamientos fueron patogénicos a los tubérculos de papa y se encontraron diferencias en patogenicidad. Se clasificaron aislamientos en PDA en tres diferentes grupos de color del micelio (B - crema brillante, P – rosa salmón, R – rosa) que variaron en patogenicidad y en nivel de crecimiento del micelio en PDA. Las colonias P mostraron el mayor daño de tubérculo, pero fueron las de crecimiento más lento en PDA. Los aislamientos mostraron varias reacciones a diferentes concentraciones de cuatro agentes de control (M – mancozeb, C – captan, CO – oxicloruro de cobre, y GE – extracto de toronja). La mayor inhibición de crecimiento de micelio (MGI) fue causada por M y la más baja por CO. Se observó fuerte MGI por GE, especialmente para los aislamientos P. Aislamientos individuales mostraron diferente susceptibilidad a los agentes de control. Se determinó la identificación de los aislamientos en ensayos de PCR con iniciadores específicos por especie FSF1/FSR1 mediante la secuenciación de fragmentos de ADN derivados de regiones ITS y la traducción del factor-1 de elongación del gen alpha (TEF). La secuencia de las regiones ITS fueron idénticas para todos los aislamientos. El análisis de los fragmentos del ADN TEF mostraron una SNP (transición C↔ T) en las secuencias de los aislamientos de los tres diferentes grupos de colores.

Keywords *Fusarium sambucinum* · Potato · Pathogenicity · Control · ITS · TEF

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Introduction

Poland is one of the largest potato producers in the world after China, India, Russian Federation, Ukraine, United States and Germany. Although its growing area has decreased in recent years, the potato is still one of the most common crops grown

in Poland (http 1- Faostat 2012; http 2- Polish Central Statistical Office 2012).

Fusarium sambucinum Fückel, teleomorph *Gibberella pulicaris* (Fries) Sacc. (Leslie and Summerell 2006) along with a few other species from the *Fusarium* genus causes dry rot, which is one of the major diseases of potato tubers that can occur both in the field and in storage, especially in North America and many parts of Europe (Boyd 1972; Desjardins 1995; Hanson et al. 1996; Hooker 1981; Peters et al. 2008; Secor and Salas 2001). Yield losses due to dry rot can be up to 25 % (Chelkowski 1989), but losses greater than 60 % of potato tubers in storage have also been reported (Stevenson et al. 2001; Theron 1991). *Fusarium sambucinum* is considered to be a toxigenic species, known to produce e.g. trichothecenes (15-monoacetoxyscirpenol and 4,15-diacetoxyscirpenol mainly), sambutoxin and enniatins (Altomare et al. 1995; Desjardins and Plattner 1989; Kim et al. 1995).

Because of the importance of potato and the extent of losses caused by *Fusarium* spp., effective protection by seed treatment and postharvest application of control substances in storage is very important (Hanson et al. 1996; Nolte et al. 2003). To develop appropriate plant breeding programs, knowledge of intraspecific diversity in the population of the fungus is also relevant (McDonald and Linde 2002).

The purpose of this study was to investigate the pathogenicity of 28 isolates of *F. sambucinum* to potato tubers, sensitivity of the fungus to control agents and diversity among the isolates including genetic features.

Materials and Methods

Collection and Isolation of *F. sambucinum*

Twenty-eight isolates of *F. sambucinum* were collected from potato tubers of different cultivars of potato (*Solanum tuberosum* L.) with dry rot disease symptoms in different Polish provinces (Table 1).

The tubers were washed in tap water, disinfected in 94 % ethyl alcohol for 1 min, washed in sterile water and shaken in 7 % H_2O_2 for 15 min and then washed three times in sterile water. To isolate the fungus 2×2 mm fragments of the tuber, from the border between healthy and diseased tissue, were cut using a sterile scalpel. After drying on sterile blotting paper, the fragments were placed on PDA, pH 5.5 (Potato Dextrose Agar, Difco, USA). After 7 days of incubation in the dark at 23 °C, pure cultures were placed first into slants with a PDA medium, next onto Petri dishes with a PDA medium and then were preliminary identified based on conidial morphology (Leslie and Summerell 2006). Single spore cultures were prepared in sterile conditions by transferring, with the use of a sterile needle, single germinated conidia obtained on 2 % water agar according to Baturo-Ciesniewska and Suchorzynska (2011). Final identification was carried out in PCR assays.

Reaction to control agents, growth and color of mycelium on PDA

Reaction of *F. sambucinum* isolates to fungicides mancozeb (M) (Dithane NeoTec 75 WG, Dow AgroSciences, Poland), captan (C) (Kaptan Zawiesinowy 50 WP, Organika-Azot, Poland), copper oxychloride (CO) (Miedzian 50 WP, Organika-Azot, Poland) and also to grapefruit extract (GE) (Biosept 33 SL, Cintamani, Poland), used to control seed piece decay or potato diseases, was tested in a radial growth assay on Petri dishes. Mycelial plugs (0.5 cm-diameter) from 10-day-old cultures were placed at the center of PDA plates amended with concentrations of 10, 50 and 100 ppm of the mentioned substances. Additionally, 330 ppm and 500 ppm of GE were tested due to the potential practical use of these higher concentrations. Growth on PDA was evaluated as a control. The experiment was carried out in three replications. Plates were incubated in the dark at 23 °C. Colony diameters were measured after 3 and 6 days post inoculation. The average daily growth of mycelium [mm] was determined and the degree of mycelium growth inhibition (MGI) was calculated in comparison to the control plates.

Also growth rate of mycelium per day measured on 3rd and 6th day after the inoculation on control dishes was used to compare isolates. On the 14th day isolates were divided into 3 main color groups: B – isolates with bright, beige mycelium, P – bright salmon pink mycelium and R – rose, carmine mycelium. It was verified if isolates classified into the three different color groups differed in growth rate, pathogenicity to potato tubers, and reaction to control agents. Also, the probability of the occurrence of each of the three colors was determined.

Pathogenicity Test

Colonies of *F. sambucinum* for infection of potato tubers were grown on Petri dishes with SNA medium for 14 days. Tubers were inoculated with a 0.5 cm disc cut from the medium with mycelium that had a high concentration of conidia. Healthy, medium-sized tubers of moderately susceptible cv. Bard were washed carefully in tap water, disinfected by immersion in 1 % NaOCl for 3 min. and washed in sterile water. After drying, a piece of tissue was cut out with a sterile cork drill (5 mm diameter and 10 mm depth), the discs with fungus were put in the hole and the removed fragment was replaced as a stopper. The pathogenicity test was carried out with three replications for each isolate. One replication was five tubers inoculated with one 0.5 cm disc of mycelium. Control samples

Table 1 Characteristics of the 28 isolates of *F. sambucinum*

Code of isolate	Origin		Colour of mycelium on PDA	Pathogenicity for potato tubers		Mean growth of mycelium on PDA	
	Potato cultivar	Location		[mm]	homoge-neous groups	[mm]	homoge-neous groups
Fsa0525[a]	Vineta	Słabomierz, N-W[b]	B[c]	20.51[d]	bcd[e]	13.39[f]	jkl
Fsa0526	Kuklik	Słabomierz, N-W	B	21.78	de	13.58	jkl
Fsa0528	Ibis	Słabomierz, N-W	P	15.45	abc	12.25	defghijk
Fsa0530	Albina	Bonin, N-W	B	20.22	bcd	8.60	a
Fsa0539	Victoria	Mielno, N-E	B	23.33	defgh	12.67	ghijkl
Fsa0540	Bila	Niewieścin, N-W	P	20.22	bcd	10.75	bcd
Fsa0542	Dorota	Zamarte, N-W	P	29.44	ij	12.63	fghijkl
Fsa0543	Asterix	Gościnno, N-W	P	78.89	k	12.03	defghij
Fsa0551	Neptun	Jadwisin, C	P	22.33	de	10.94	bcde
Fsa0552	Irga	Jadwisin, C	P	21.00	cd	11.54	cdefgh
Fsa0553	Kuklik	Mrocza, N-W	P	22.45	de	12.88	hijkl
Fsa0555	Ibis	Jankowo, N-W	B	28.33	fghi	12.54	efghijkl
Fsa0557	Bila	Zamarte, N-W	B	28.78	hij	12.50	efghijkl
Fsa0637	Gracja	Kruszyn Kraj., N-W	P	34.00	j	9.39	ab
Fsa0709	Orlik	Osiny, S-E	B	32.00	ij	13.42	jkl
Fsa0718	Drop	Osiny, S-E	B	22.11	de	13.92	l
Fsa0719	Gracja	Osiny, S-E	P	28.22	fghi	9.38	ab
Fsa0720	Korona	Osiny, S-E	R	20.67	bcd	13.22	ijkl
Fsa0726	Vineta	Włościbórz, N-W	B	10.56	a	11.00	bcdef
Fsa0727	Denar	Wiśniewka, N-W	R	23.00	defg	12.15	defghijk
Fsa0730	Umiak	Gozdanin, N-W	B	29.11	ij	11.64	cdefghi
Fsa0731	Hinga	Twierdzeń, N-W	B	23.45	defgh	13.78	kl
Fsa0732	Innowator	Słupsk, N	R	22.78	def	12.69	ghijkl
Fsa0734	Lord	Zamarte, N-W	B	18.11	bcd	11.61	cdefghi
Fsa0736	Kuba	Zamarte, N-W	R	15.22	ab	11.03	cdef
Fsa0737	Bard	Mochełek, N-W	R	12.44	a	10.35	bc
Fsa0738	Rosalind	Mochełek, N-W	R	26.89	efghi	11.22	cdefg
Fsa0740	Korona	Mochełek, N-W	R	28.44	ghij	13.17	hijkl
control	/	/	/	9.22	/	/	/
mean	/	/	/	24.99	/	11.94	/
mean for B	/	/		23.19	/	12.39	/
mean for P	/	/		30.22	/	11.31	/
mean for R	/	/		21.35	/	11.98	/

Abbreviations and designations in table

[a] The first two digits in an isolate code indicate the year of isolation, the last two digits indicate the number of the isolate in the collection from a given year; [b] N-W - north-western Poland. C - central Poland. N-E - north-eastern Poland. S-E - south-eastern Poland. N - northern Poland; [c] B - bright-beige, P - salmon pink, R - rose; [d] mean value of rot diameter and depth; [e] The homogeneous group obtained by comparing the mean values using LSD; [f] The mean growth per day calculated based on colony diameter measured after 3 and 6 days from the inoculation on PDA

were tubers inoculated with SNA medium disc without fungus. Tubers in plastic cuvettes were incubated in a growing chamber at 12–14 °C, moisture 90–95 % for 30 days. In the case of each tuber the diameter and depth of rot were measured in mm. The mean value of those dimensions minus the mean value from the control samples were the basis for determining the pathogenicity of the isolates.

Statistical Analysis Applied in Pathogenicity and Petri Dish Tests

To compare the pathogenicity of isolates one-way analysis of variance (ANOVA) was applied. The calculations were made for the level of $\alpha = 0.05$, in Matlab ver. 7.9 with the Statistics Toolbox. In the Petri dish tests the statistical analyses ANOVA

or the nonparametric test of Kruskal-Wallis (KW) was carried out. The calculations were made for the level of α =0.05, in Matlab ver. 7.9 program with Statistic Toolbox version 6.1. In the KW test, values were converted to ranks. In the case of significant differences Tukey's test was used to determine the least significant difference criterion LSD for α =0.05. Chi-squared test for α=0.05 was applied to determine whether the occurrence of any of the three colors of mycelium is more probable. When analyzing the effect of the 4 control agents on MGI the statistical calculations were conducted additionally based on the average values calculated from the three concentrations (10, 50 and 100 ppm). Separate tests were performed for the two additional, higher, concentrations of GE.

To determine the relationships between the pathogenicity of isolates for the potato tubers and the growth on PDA and level of MGI by control agents, Pearson's correlation coefficient with a corresponding probability (if p <0.05 the correlation exists) was calculated.

Extraction of DNA and General PCR Conditions

DNA was extracted from 4-day-old mycelium grown on Potato Dextrose Broth (PDB; Difco, USA) according to the Cetyl Trimethyl Ammonium Bromide (CTAB) modified method of Doyle and Doyle (1990). DNA was diluted to 20 ng·μl^{-1} in water and stored at −20 °C until use.

Each 12.5 μl PCR reaction contained 0.2 mM of dNTPs, 1× buffer, 1× Q solution, 1 mM $MgCl_2$, 0.5U of *Taq* DNA polymerase (PCR Core Kit QIAGEN, USA), 0.6 pM of each primer and 50 ng of DNA. The amplification process involved an initial denaturation of 2 min at 95 °C, followed by 30 or 35 cycles at 95 °C for 30 s, annealing temperature and extension time specific for each assay, 72 °C for 1 min, a final extension at 72 °C for 5 min, and a 4 °C hold. The amplified PCR products were electrophoresed on 1.4 % agarose gel with a TBE running buffer stained with ethidium bromide. A molecular marker of 100 bp (EURx, Poland) was used. The results were scanned into a computer imaging file with a gel documentation system with a digital camera (INTAS, Germany).

Species Identification and Determination of Potential Mycotoxigenic Ability in PCR Assays

To confirm microscopic identification of *F. sambucinum,* the species-specific primer pair FSF1/FSR1 was used (Mishra et al. 2003). Additionally, ITS region and partial translation elongation factor 1-alpha (TEF) sequence were analyzed. The primer pair ITS1/ITS4 (White et al. 1990) was used for the amplification of ITS regions containing the 5.8S rDNA. The ef1/ef2 (Geiser et al. 2004) and ef1'/ef2' (Du et al. 2012) were used for amplification of the TEF gene region. As a control, in TEF assay, different *Fusarium* species such as *F. avenaceum* (2 isolates), *F. culmorum* (4), *F. equiseti* (1), *F. garminearum* (2), *F. langsethiae* (2), *F. oxysporum* (2), *F. poae* (1) *F. solani* (3) and *F. sporotrichioides* (1) originated from different plants, from our own collection, were used. The PCR products amplified with these three primer pairs were sequenced (Genomed, Poland). Sequence comparisons were carried out using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). The potential ability to produce trichothecenes was tested using primers specific for the gene *Tri*5 and for the gene *Tri*4 that determines production of A-type trichothecenes (Nicholson et al. 2004). Sequences of all primers (IBB PAN, Poland), product sizes, annealing temperature and extension time for each assay are presented in Table 2.

Results

Reaction to Control Agents, Growth and Color of Mycelium on PDA

Isolates showed differentiated reactions to 4 control agents and their concentrations (Table 3).

Significant differences in their effect on MGI were found, p=0 (Table 4 A). Effectiveness in MGI was observed especially in the case of M, but also with GE and C. The lowest MGI was caused by CO. In the case of the 10 ppm concentration, M suppressed the growth of mycelium by 36.1 %, GE by 28.7 %, while C only by 9.7 % and CO by 4.5 %. The differences in the mean effect of the control agents and their concentrations are shown in Fig. 1. Increasing the concentration of C resulted in raising its efficacy in comparison to GE. The most significant increase of MGI, from 9.7 to 52.1 %, was observed after increasing C concentration from 10 ppm to 50 ppm. Further increasing to 100 pm did not cause significantly greater inhibition. A similar result was also observed for M (Table 3).

In some cases more intensive growth of the fungus was observed (negative MGI) in comparison to the control on a medium with the addition of a control agent. Generally, the lowest concentration of each substance caused significantly weaker MGI in comparison to higher concentrations of each control agent. The exception was CO. Although no significant differences between efficacy of the three concentrations of CO were found, some trends were noticed: the concentration of 50 ppm inhibited the mycelium growth at the weakest level in comparison to 10 and 100 ppm. In some cases the inhibition effect of CO was the strongest on plates with the lowest concentration (e.g. for Fsa0526 or Fsa0731). CO, regardless of concentration, only slightly affected MGI. Even the highest (100 ppm) concentration of CO in five isolates either did not cause growth inhibition or even promoted growth (e.g. Fsa0734) (Table 3).

Table 2 Details of amplification conditions including sequences of primers, annealing temperature, extension time and cycle number

Target	Primers	Sequence 5'→3'	PCR conditions	Product size (bp)
F. sambucinum	FSF1/FSR1	ACATACCTTTATGTTGCCTCG GGAGTGTCAGACGACAGCT	58 °C, 30 s, 30 cycles	315
TEF	ef1/ef2	ATGGGTAAGGA(A/G)GACAAGAC GGA(G/A)GTACCAGT(G/C)ATCATGTT	53 °C, 55 s, 30 cycles	~700
TEF	ef1'/ef2'	ATGGGTAAGGAAGACAAGAC GGAGGTACCAGTGATCATGTT	58 °C, 55 s, 30 cycles	~700
ITS	ITS1/ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	52 °C, 50 s, 35 cycles	~550
*Tri*5	Tri5F/Tri5R	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCTG	60 °C, 30s, 30 cycles	545
*Tri*4	T4F1506/T4EndR2	CCCCTGGCTACTCTCGAGA AAGCTTTGAGAACCTTCAC	54 °C, 30s, 30 cycles	550

Use of the 330 ppm concentration generally, with the exception of 4 isolates, resulted in an increase of MGI with a 100 % of MGI being observed for 3 isolates. The concentration of 500 ppm increased mean MGI and 100 % of MGI was noted for 8 isolates. Only isolate Fsa0737 did not react positively to an increase in the concentration of GE above 100 ppm (Table 3).

Individual isolates showed significantly (p=0) different susceptibility to specific substances (Table 3; Table 4 B,C,D; Fig. 2,3,4). Some isolates were more strongly inhibited by the lowest concentration of GE than by chemical (synthetic) substances at the same concentration e.g. Fsa0551, Fsa0528, Fsa0555, Fsa0718 or Fsa0732. Most of the isolates were inhibited slightly more strongly by M than by C. For some isolates (Fsa0530 and Fsa0734) C was more effective, excluding the concentration of 10 ppm, which generally only weakly inhibited growth of all the isolates. An interesting reaction was shown by isolate Fsa0734: concentration of 10 ppm of C caused its stronger growth, than on the medium without the control agents (−14.8 %), while the further increasing to 50 and 100 ppm resulted in a strong inhibition that was similar to the reaction of the other isolates. This isolate also grew the most intensively on the medium with the addition of any concentration of CO (Table 3).

Based on observation of mycelium on the control plates the isolates were divided into three color groups. Bright-beige (B) group was dominant (12 isolates – 42.9 %), 9 isolates were salmon pink (P) and 7 rose (R). However, Chi-squared test showed that there was no color significantly predominant. In the isolates from 2007 the red color of mycelium was predominant and all red isolates were collected in this year.

The significant differences in mycelium growth rate on PDA between the 28 isolates were shown in an ANOVA test. Growth rate varied from 8.6 mm (Fsa0530) to 13.9 mm (Fsa0718), with a mean value of 11.9 mm. These two isolates formed two distinct homogeneous groups, respectively *a* and *l*. The rest of the isolates were assigned to intermediate groups between those two. The ANOVA test also showed that the 3 different color groups of isolates grew significantly differently. B, R and P colonies formed three homogeneous groups, respectively *a*, *ab* and *b*. The P colonies, which caused the greatest tuber damage, grew the slowest (Table 1, Table 4 E, F).

Isolates of a different color of mycelium showed different sensitivity to the control agents (Table 3, Table 4 G, Fig. 5). Significant differences were found in reaction to M, where R isolates were more strongly inhibited than P and B isolates. Simultaneously R isolates were the most resistant to C and GE. Isolates of B group, compared with groups P and R, reacted much less to CO.

When considering the five concentrations of GE, significant differences in the MGI were noted. P was inhibited most strongly, and R the least. With increasing concentration of GE (from 10 ppm to 500 ppm) P and B isolates were increasingly inhibited, while the R isolates did not show any further increase of growth inhibition with concentrations greater than 100 ppm. Increasing the GE concentration to 500 ppm resulted in mean MGI for P isolates at the level of 97 % (Table 3).

Pathogenicity Test

All isolates were pathogenic to potato tubers cv. Bard and significant differences in pathogenicity were revealed, p=0. Mean size of tuber damage was 25 mm, ranging from 10.6 mm (Fsa0726) to 78.9 mm (Fsa0543). These two isolates formed two distinct homogeneous groups, respectively *a* and *k*. Others isolates were assigned to intermediate groups. Some differences in the pathogenicity of isolates differing in color were noted, but the KW test showed that they were not statistically significant (p=0.176) (Table 1).

Based on the Pearson correlation coefficient it was found that there was a correlation between pathogenicity for potato tubers and MGI on PDA for the highest GE concentration (500 ppm). The correlation coefficient was

Table 3 Mycelium growth inhibition (MGI) by control agents on PDA in Petri dishes experiment [%]

Code of isolate	Mancozeb			Captan			Copper oxychloride			Grapefruit extract				
	10[a]	50	100	10	50	100	10	50	100	10	50	100	330	500
Fsa0525	47.5[b]	60.1	63.7	6.4	56.6	61.0	4.1	2.0	14.9	30.0	38.0	49.0	66.1	69.8
Fsa0526	47.0	60.3	62.5	8.5	55.5	58.9	17.6	7.1	11.1	30.8	50.5	58.7	72.2	78.1
Fsa0528	9.9	58.1	64.5	11.8	52.2	51.8	−6.6	−3.8	0.1	28.8	58.3	65.5	70.8	73.5
Fsa0530	26.4	39.0	46.5	13.9	62.2	66.0	1.1	−11.2	−16.5	28.6	42.3	45.0	90.0	90.0
Fsa0539	18.7	56.9	63.5	0.6	48.6	47.8	−9.3	−11.4	−2.8	23.0	61.9	68.4	64.8	70.5
Fsa0540	47.2	57.9	59.7	13.6	52.5	56.6	−11.2	9.0	14.0	39.1	35.4	50.4	70.1	100
Fsa0542	27.9	53.9	57.1	2.8	52.7	57.2	1.1	8.6	2.4	30.4	47.9	56.8	100	100
Fsa0543	45.7	54.6	56.1	10.0	59.3	58.8	11.7	5.7	19.0	29.3	57.8	56.1	78.4	100
Fsa0551	22.4	54.0	56.2	12.3	54.8	64.0	−1.5	6.4	5.7	37.6	54.8	63.4	100	100
Fsa0552	47.2	59.0	58.6	19.7	64.4	66.0	24.7	17.3	30.3	31.7	55.9	61.1	92.5	100
Fsa0553	51.3	57.1	57.0	16.8	56.0	62.4	24.3	14.8	25.3	34.6	44.6	56.1	79.8	100
Fsa0555	16.2	58.3	68.6	14.4	55.1	57.3	−1.0	8.1	6.9	33.9	44.7	49.2	73.7	78.9
Fsa0557	41.3	61.1	69.8	17.2	64.4	67.8	22.4	−3.9	21.1	33.1	41.3	51.4	70.5	74.8
Fsa0637	44.5	54.6	53.4	7.6	53.8	54.2	−0.4	1.3	3.7	26.5	43.0	50.2	71.7	100
Fsa0709	38.6	66.3	62.1	6.8	56.2	57.8	14.0	−0.4	10.1	32.0	39.7	51.0	68.9	76.0
Fsa0718	5.6	34.1	38.2	7.0	42.3	31.9	−0.2	−8.6	−15.4	30.0	43.1	50.0	54.1	57.5
Fsa0719	45.9	49.9	53.2	3.9	50.5	56.6	−2.3	1.3	12.9	34.9	40.9	49.1	100	100
Fsa0720	47.4	66.1	68.8	5.0	52.8	56.2	14.1	6.5	15.7	26.4	43.6	50.3	53.3	56.5
Fsa0726	38.5	45.6	49.3	24.9	52.9	58.1	−14.6	−7.9	−6.8	39.4	40.0	46.3	59.5	57.2
Fsa0727	47.3	64.8	67.6	11.0	45.9	49.4	1.7	6.0	6.3	20.8	58.2	62.1	59.2	61.5
Fsa0730	39.5	54.2	62.8	8.0	38.6	42.2	3.5	0.0	9.1	24.5	30.6	38.0	57.8	63.6
Fsa0731	40.4	64.8	66.6	8.5	48.7	57.3	20.7	14.2	12.9	19.8	55.7	70.9	52.3	59.3
Fsa0732	7.1	63.3	65.4	5.3	47.0	50.1	1.1	3.6	15.4	26.2	43.0	48.4	56.8	58.3
Fsa0734	15.7	13.2	23.6	−14.8	50.7	54.2	−22.7	−17.7	−31.6	12.6	33.0	35.6	72.0	75.9
Fsa0736	54.1	64.3	68.5	15.1	46.6	46.9	−5.7	−4.2	4.1	12.7	27.2	40.6	47.8	47.7
Fsa0737	45.4	59.7	62.6	10.5	45.8	49.8	7.3	9.0	0.1	32.6	50.9	56.6	52.2	50.1
Fsa0738	34.7	54.7	60.5	9.5	39.3	44.1	10.1	4.8	4.8	29.4	40.4	46.4	55.6	54.5
Fsa0740	56.3	69.1	73.8	15.5	52.5	57.7	21.5	5.1	19.5	25.7	38.8	44.1	65.0	64.5
mean	36.1	55.5	59.3	9.7	52.1	55.1	4.5	2.2	6.9	28.7	45.1	52.5	69.8	75.6
mean for B	31.3	51.2	56.4	8.5	52.6	55.0	3.0	−2.5	1.1	28.1	43.4	51.1	66.8	71.0
mean for P	38.0	55.5	57.3	10.9	55.1	58.6	4.4	6.7	12.6	32.5	48.7	56.5	84.8	97.1
mean for R	41.7	63.1	66.7	10.3	47.1	50.6	7.2	4.4	9.4	24.8	43.2	49.8	55.7	56.1

[a] concentration of the substance in ppm, [b] mean value of 3 replications

Abbreviations and designations in table: B - bright-beige mycelium, P - salmon pink mycelium, R - rose mycelium

0.413 (p=0.029). This means that the higher the pathogenicity of an isolate (measured by the growth on potato tubers), the greater the MGI of this isolate for this concentration of GE. There was no correlation between pathogenicity for potato tubers and growth of isolates on PDA. The correlation coefficient was 0.077 (p=0.697). Some isolates growing very strongly on the PDA did not cause severe damage to tubers. The most pathogenic isolate Fsa0543, which caused damage to the tubers at the level of 78.89 mm in the medium reached a diameter of 12.03 cm, while isolate Fsa0718, which caused damage of the tubers of only 22.11 mm grew the most and reached 13.92 mm on PDA medium.

PCR Assay

PCR assay with species specific primers FSF1/FSR1 confirmed microscopic analysis. In the case of all 28 isolates product of 315 bp was obtained.

NCBI Blast analysis of the 674 - bp segment of *tef*1 gene and the 534 - bp segment of ITS region also confirmed the species of *F. sambucinum*. Sequences of three isolates

Table 4 Summary of ANOVA and Kruskal-Wallis (KW) analyses for 28 isolates of *F. sambucinum*

Source of variation	SS	df	MS	F (ANOVA)/ Chi-sq (KW)	Prob>chi-sq
A. Mean effect of 4 control agent and their concentrations on MGI (KW) (Fig. 1)					
Groups	5.1755e+008	11	47049965.1	1527.36	0
Error	1.65238e+008	2004	82453.9		
Total	6.82787e+008	2015			
B. Effect of 10 ppm concentrations of control agents on MGI of individual isolate on PDA (KW) (Fig. 2)					
Groups	2.14892e+007	111	193,596	570.21	0
Error	3.79849e+006	560	6,783		
Total	2.52876e+007	671			
C. Effect of 50 ppm concentrations of control agents on MGI of individual isolate on PDA (KW) (Fig. 3)					
Groups	2.27992e+007	111	205398.1	604.96	0
Error	2.48897e+006	560	4444.6		
Total	2.52882e+007	671			
D. Effect of 100 ppm concentrations of control agents on MGI of individual isolate on PDA (KW) (Fig. 4)					
Groups	2.13422e+007	111	192,272	566.29	0
Error	3.94625e+006	560	7046.9		
Total	2.52884e+007	671			
E. Mycelium growth on PDA (ANOVA) (Table 1)					
X1	314.839	27	11.6607	5.68	2.48745e-012
Error	287.56	140	2.054		
Total	602.399	167			
F. Mycelium growth on PDA in respect to mycelium color (ANOVA) (Table 1)					
X1	35.938	2	17.969	5.23	0.0063
Error	566.461	165	3.4331		
Total	602.399	167			
G Mean effect of control agents on MGI in respect to mycelium color (KW) (Fig. 5)					
Groups	3.29607e+008	11	29964267.1	972.72	0
Error	3.5318e+008	2004	176237.6		
Total	6.82787e+008	2015			

differing in the color of mycelium were deposited in GenBank with Accession No. KC899118 - KC899120 for *Tef1α* gene and KC899115 - KC899117 for ITS regions.

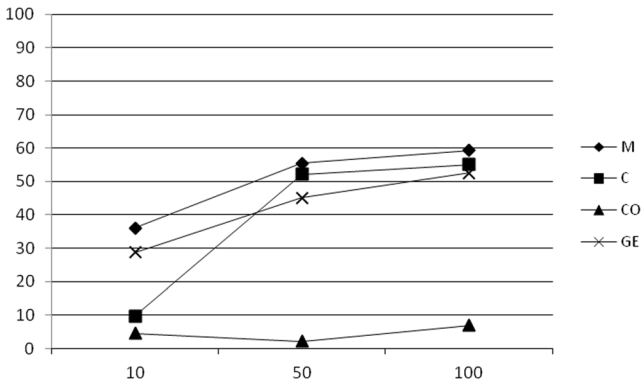

Fig. 1 Effect of 10, 50 and 100 ppm concentrations of Mancozeb, Captan, Copper oxychloride and Grapefruit extract on mycelium growth inhibition on PDA [%]

Analysis of the TEF PCR product generated using the ef1/ef2 primers showed a difference in the sequences of isolates differing in color. There was one single nucleotide polymorphism (SNP) between the sequences of the groups of isolates. It was located at position 390 (transition C ↔ T) of the amplicon. In the case of the B and P isolates thymine was identified, while cytosine was found in R isolates (Fig. 6).

Products with ef1/ef2 primers were obtained not only for *F. sambucinum*, but also for all control samples from *Fusarium* spp. The use of ef1'/ef2' primers resulted in product being obtained for a smaller number of control isolates. It resulted in product presence for *F. poae*, but not for all isolates of *F. solani*, *F. oxysporum* and *F. langsethiae*. Product was missing for *F. avenaceum*, *F. culmorum*, *F. eqiuseti*, *F. graminearum* and *F. sporotrichioides*. Sequence of the ITS regions were identical for all DNA samples regardless of their color.

*Tri*5 and *Tri*4 genes were present in all isolates, which shows their potential ability to produce A-type trichothecenes. HPLC analyses of randomly chosen potato tubers infected

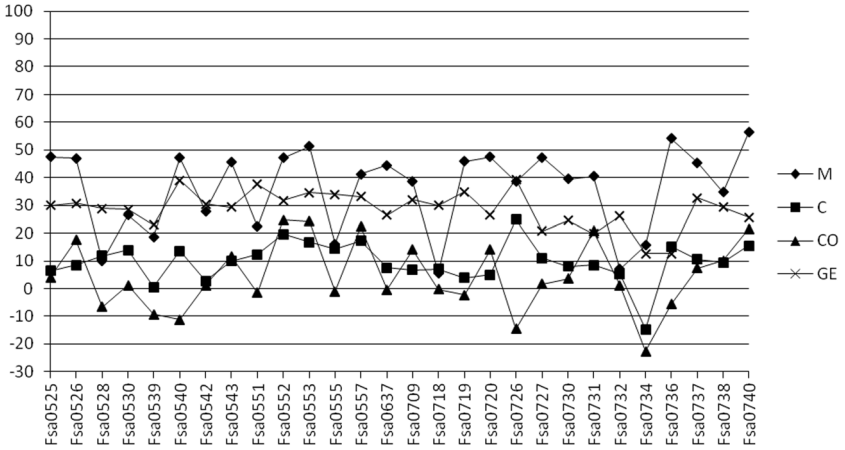

Fig. 2 Effect of 10 ppm concentrations of Mancozeb, Captan, Copper oxychloride and Grapefruit extract on mycelium growth inhibition of single isolate on PDA [%]

with different isolates in pathogenicity test showed the presence of monoacetoxyscirpenol (MAS) and diacetoxyscirpenol (DAS) in their tissues. Concentration of mycotoxins was not associated with the size of the tuber rot (data not presented).

Discussion

According to the literature the precise classification of isolates of *Fusarium* spp. causing dry rot of potato tubers has been problematic. The treatment of *F. sambucinum*, one of the causal agent of the disease, differs depending upon the authority (Leslie and Summerell 2006) because of contradictory classification systems proposed by various researchers e.g. Booth (1971), (Gerlach and H. Nirenberg 1982), Nelson et al. (1983) and Nirenberg (1995) that are primarily based on morphological characters of the colonies. For instance (Nelson et al. 1983) included *F. sulphureum* and other forms in a broad species concept of *F. sambucinum* sensu *lato*. Nirenberg (1995) divided *F. sambucinum* sensu lato into three taxa: *F. sambucinum* Fuckel sensu stricto, *F. venenatum* Nirenb. and *F. torulosum* (Berk. & Curt.) Nirenb. RAPD assays of Yoder and Christianson (1998) support Nirenberg's contention that the three species are indeed distinct. Leslie and Summerell (2006) noted they can be confused because of their very similar morphology. Molecular methods have greatly facilitated identification of the species. Species specific primers (Mishra et al. 2003) allowed for a clear identification of our isolates and classified them into *F. sambucinum*.

Sequencing of ITS as well TEF regions confirmed SCAR-PCR assay. ITS regions are commonly used for identification of *Fusaria* (Tan and Niessen 2003; Wilson et al. 2004). Sometimes clear identification is not possible due to the fact that some species have identical sequences of some ITS regions, e.g. six species analyzed by Waalwijk et al. (1996) had identical ITS1 sequences. However, O'Donnell (1992) noted that the rDNA genic regions are highly conserved within *F. sambucinum,* which probably in the case of this species allowed him to obtain a reliable result. Schoch et al. (2012) evaluated six DNA regions as potential DNA barcodes for Fungi. They concluded that the ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. NCBI Blast analysis of ITS regions of our isolates clearly showed the species *F. sambucinum,* because of the highest level of identity with

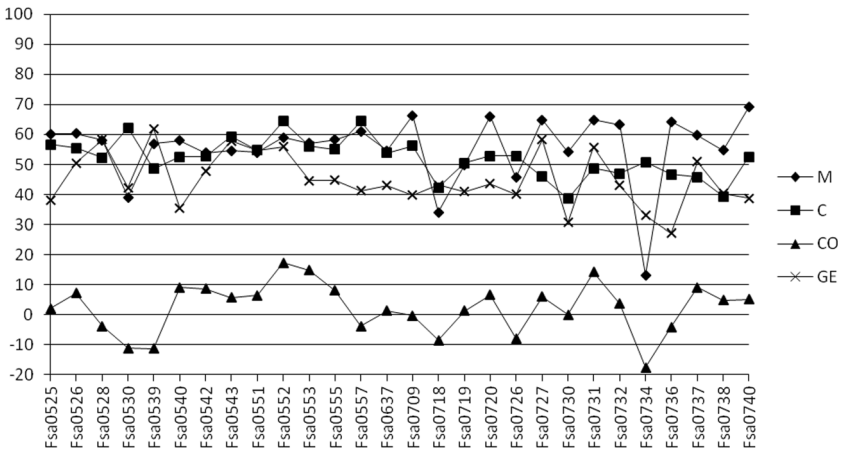

Fig. 3 Effect of 50 ppm concentrations of Mancozeb, Captan, Copper oxychloride and Grapefruit extract on mycelium growth inhibition of single isolate on PDA [%]

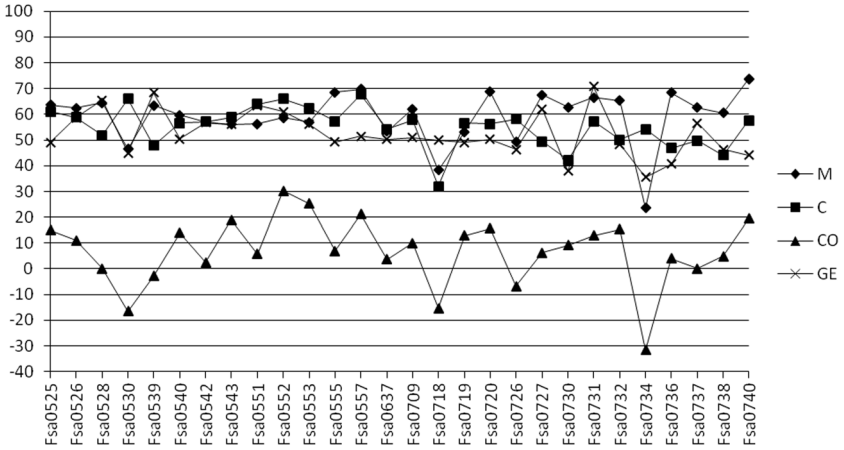

Fig. 4 Effect of 100 ppm concentrations of Mancozeb, Captan, Copper oxychloride and Grapefruit extract on mycelium growth inhibition of single isolate on PDA [%]

isolates described and deposited in GenBank e.g. by (O'Donnell 1992) and Hatsch et al. (2004). Also, Wharton et al. (2006) based on the sequences of the ITS region identified isolates of *F. sambucinum* causing potato tuber sprout rot.

Due to the fact that many *Fusaria* within the *Gibberella* clade possess non-orthologous copies of the ITS2, which can lead to incorrect phylogenetic inferences (O'Donnell and Cigelnik 1997; O'Donnell et al. 1998) in many cases analysis of the translation elongation factor 1-*a* (TEF) gene is applied. TEF encodes an essential part of the protein translation machinery and has high phylogenetic utility because it is highly informative at the species level in *Fusarium*. Non-orthologous copies of the gene have not been detected in the genus and universal primers have been designed that work across the phylogenetic breadth of the genus (Geiser et al. 2004). PCR assays with universal primers ef1/ef2 and ef1'/ef2' that amplify TEF sequences allowed us to obtain the product with many species of *Fusarium*. (Geiser et al. 2004) using the ef1/ and ef2 primers generated products with numerous *Fusarium* species, including all isolates applied as control samples in our studies, and created FUSARIUMID v. 1.0 database of partial translation elongation factor 1-alpha (TEF) DNA sequences.

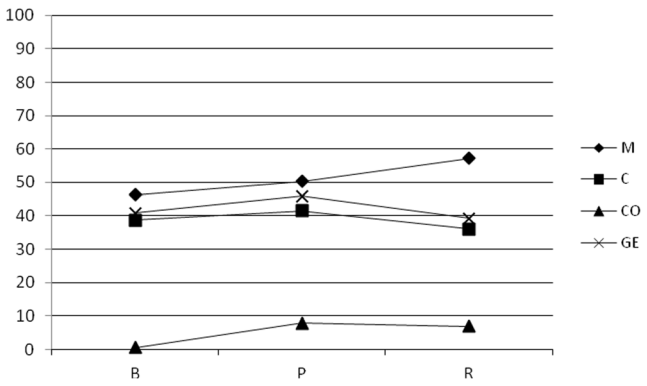

Fig. 5 Mean effect of three concentrations (10, 50 and 100 ppm) of Mancozeb, Captan, Copper oxychloride and Grapefruit extract on mycelium growth inhibition in respect to mycelium color (B - bright-beige, P - salmon pink and R - rose) [%]

The use of primers ef1'/ef2' (Du et al. 2012) reduced the *Fusarium* species for which the product was obtained. Unlike our results, (Du et al. 2012) obtained amplicons e.g. for *F. avenaceum* and *F. equiseti*. Since amplicons of different species of *Fusarium* are obtainable with these primer pairs, it is not possible to identify the species of *F. sambucinum* based only on the presence of the product (band) and only sequence analysis allows for the identification of the species. The sequence of our R isolates was identical with *F. sambucinum* sequences AJ543602- AJ543605 (Kristensen et al. 2005) and B and P differ only with SNP. Differences between our sequences and the sequences of *F. torulosum* and *F. venenatum* were much higher.

Differences in TEF gene sequence for one single nucleotide polymorphism were found between our isolates differing in color. Wollenweber and Reinking (1935) distinguished several forms of *F. sambucinum* based primarily on colony color and other morphological traits. According to Booth (1971) pigmentation of mycelium is often characteristic for different forms and species and could be an important feature used to distinguish them. He considered *F. sambucinum* to be a few distinct species including *F. sambucinum* represented by the red cultures, *F. sulphureum* the yellowish and *F. trichothecioides* the rose colored, pionnotal ones. However, the color of mycelium does not differentiate between the species most commonly confused with *F sambucinum* sensu stricto, ie *F. torulosum* and *F. venenatum* (Desjardins and Beremand 1987; Logrieco et al. 1995). Our studies agree with these observations of the mentioned authors who noted isolates of different pigment (red, yellow, rose, brown) within the same species, *F. sambucinum* sensu stricto. Also, mycelium of *F. torulosum* can be red brownish or beige (Logrieco et al. 1995).

Mycotoxin profiles can also be useful in *Fusarium* spp. identification (Altomare et al. 1995; Logrieco et al. 1995; Thrane and Hansen 1995). Desjardins and Plattner (1989) revealed that production of trichothecene toxins is a common trait of *G. pulicaris* strains isolated from potato tuber dry-rot.

Fig. 6 Alignment of *Tef1α* gene regions for the isolates differ in color of mycelium

They, as well as (El-Banna et al. 1984), found that trichothecenes were formed in potato tubers infected with *F. sambucinum*. Jelen et al. (1995) and Ellner (2002) detected A-type trichothecenes (e.g. DAS, MAS) in all potato tubers inoculated with *F. sambucinum*. The detection of *Tri*5 and *Tri*4 genes indicated the ability of our isolates to produce trichothecenes, which was confirmed in our studies by randomly carried out HPLC analyses (data not published). Hohn and Desjardins (1992) detected and identified the *Tri*5 gene in *F. sambucinum*. This gene initiates the trichothecene biosynthetic pathway. Edwards et al. (2001) and Nicholson et al. (2004) confirmed by PCR assay the ability of *F. sambucinum* to produce Type-A trichothecenes, which is related to gene *Tri*4. The presence of these substances in the tuber poses a threat not only to the plants, but mainly to the consumer, as these are some of the most toxic mycotoxins and are more acutely toxic than the B trichothecenes (Ueno 1983). *Fusarium sambucinum* is able to produce trichothecenes, unlike e.g. *F. torulosum* (Altomare et al. 1995). Molecular analyses showed that all of our isolates are genetically able to produce A-group trichothecenes, which therefore excludes the presence of *F. torulosum*, which is capable of producing moniliformin. Trichothecene production is related to pathogenicity (Desjardins and Hohn 1997; Wagacha and Muthomi 2007). Mycotoxins have been found in tuber tissues showing symptoms of tuber dry rot by Peters et al. (2008). For an evaluation of the pathogenicity of the isolates we measured, as Valluru et al. (2006), the diameter and depth of the lesions in the potato tuber. In many of the isolates analyzed in our experiment we observed extensive damage to potato tubers of cv. Bard. We also showed that all our isolates were pathogenic and that they varied in pathogenicity. It is consistent with observations of Desjardins and Hohn (1997) who revealed that pathogenicity of G. *pulicaris* can vary and also that the reaction of potato cultivars to different isolated can be variable. Isolates can range from highly pathogenic isolates to completely nonpathogenic isolates (El-Hassan et al. 2007). In addition to varying pathogenicity measured by the mycelium development in potato tubers, isolates differed in growth on PDA medium. No correlation between these parameters indicates that the measurement of the growth rate of the fungus on a medium containing an extract of potato tubers is not passed through to the development of the mycelium in the tuber and the related degree of pathogenicity to tubers.

In order to limit the development of fungi of the genus *Fusarium*, including *F. sambucinum*, on potato tubers various control agents have been used for many years, because no high level resistance to the pathogens exists among commercially grown cultivars (Bojanowski et al. 2013). Control of dry rot has been achieved primarily by applications of thiabendazole (TBZ) (Powelson et al. 1993; Secor and Gudmestad 1999). However, a tolerance and resistance of *F. sambucinum* strains to TBZ has been found (Desjardins et al. 1993; Gachango et al. 2012; Hanson et al. 1996).

Analyses of Bojanowski et al. (2013) have shown that different fungicides in varying degrees may control potato dry rot. Mancozeb, recommended for potato seed treatment, that has proven effective in the reduction of the pathogen after application on seed potato tubers (Cwalina-Ambroziak and Czajka 2006; Leach and Nielsen 1975), also limited the growth of our *F. sambucinum* isolates. Captan as well clearly inhibited the growth of *F. sambucinum*. Both preparations have also been found to be effective in reducing the severity of potato rot caused by *F. solani* and, as in our study, mancozeb was again somewhat more effective (Taskeen-Un et al. 2011). However, while captan effectively inhibited growth of our isolates at the higher concentrations, the use of the lowest concentration in the case of one of the isolates stimulated its growth, which suggests that excessive dose reduction may be even more dangerous than no use of the fungicide. Taskeen-Un et al. (2011) found that higher concentration of fungicides caused more reduction in the rot severity caused by *F. solani* than did lower concentrations. Similarly, in our Petri dish experiments, such an effect on *F. sambucinum* isolates was particularly evident in the case of increasing the concentration from 10 to 100 ppm.

The reaction of our isolates to control agents varied. Such differentiation was also observed by Daami-Remadi et al. (2006) and Choiseul et al. (2007) for various fungi causing dry tuber rots. They noted that the changes in isolate sensitivity to fungicides they recorded highlight the need for regular monitoring programs to be conducted in order that disease-control strategies can remain effective

Bojanowski et al. (2013) also noted that apart from the use of fungicides, which has been the primary disease management practice in potato production (Hamm et al. 2008), several biological control agents were effective against *F. sambucinum* and could be used as an alternative to synthetic chemical compounds. The global trend appears to be shifting towards a reduced use of fungicides. There is a strong public and scientific desire to develop effective methods of controlling postharvest diseases that are perceived as safe by the public and pose reduced risk to human health and the environment (Mari et al. 2007; Wisniewski and Wilson 1992). To such eco-friendly control agents can be included, for example, citrus extracts that show an effective antimicrobial activity

against foodborne pathogens including bacteria and fungi (Fisher and Phillips 2008). In Poland, the grapefruit extract 'Biosept' is approved for use in organic agricultural systems. It stimulates the resistance of plants and works as a fungicide (Orlikowski and Skrzypczak 2003). We observed its high efficacy in reducing the growth of *F. sambucinum* on PDA. It worked only slightly less well than mancozeb or Captan at comparable concentrations, and increasing the concentration to 500 ppm in some cases definitely inhibited the growth of some isolates. Because of the lack of toxicity of this substance, it seems to be important to think about the potential control of *F. sambucinum* with grapefruit extract, including postharvest damage, especially since the salmon pink colonies, which caused the greatest tuber damage, were most strongly inhibited by Biosept. In agreement with our experiments with grapefruit extract, increasing the concentration of essential oil extracted from the epicarp of *Citrus sinensis* to a few hundred ppm significantly improved fungitoxicity against 10 post-harvest pathogens of fruits (Sharma and Tripathi 2006). Bevilacqua et al. (2012) noted a reduction of *F. oxysporum* spores in pineapple juice after the application of citrus extract. Biosept greatly limited pathogens of barley in organic farming (Baturo 2009) and showed wide fungistatic activity in the control of leaf and soil-borne pathogens including *Fusarium* spp. (Orlikowski and Skrzypczak 2003; Patkowska 2006).

Apart from the grapefruit extract also copper products, including copper oxychloride, are applied in organic farms in Poland against e.g. potato blight (http 3- IOR). The Organic Farming Research Foundation organic farmer survey reported that copper is the second, after sulfur, most-applied disease control material on organic farms in the USA (Andrews and B. Baker 2013). Our studies revealed not only its ineffectiveness in inhibiting the growth of *F. sambucinum,* but that it can even stimulate the growth of the pathogen. This suggests that the use of this control agent against potato dry rot caused by this pathogen could be inefficient.

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